Consumer and Corporate Affairs Canada | Consommation et Corporations Canada

(11) (A) No. 1 143 656

(45) ISSUED 830329

(52) CLASS 167-247

(51) INT. CL.[3] A61K 37/22

(19) (CA) **CANADIAN PATENT** (12)

(54) LIPOSOME INCLUDING ACTIVE SUBSTANCE

(72) Watanabe, Kozyu,
Japan

(73) Granted to Kureha Kagaku Kogyo Kabushiki Kaisha
Japan

(21) APPLICATION No. 351,077

(22) FILED 800501

(30) PRIORITY DATE Japan (54283/79) 790502

No. OF CLAIMS 6

Canada

DISTRIBUTED BY THE PATENT OFFICE, OTTAWA
CCA-274 (11-82)

LIPOSOME INCLUDING ACTIVE SUBSTANCE

ABSTRACT OF THE DISCLOSURE

Liposome prepared by using a phospholipid and oily substance as a base material for the liposome including an active substance in the vesicle of the liposome is disclosed, the liposome having a wall membrane of high strength and being excellent in slowly releasing the included active substance therewithin to outside therefrom.

BACKGROUND OF THE INVENTION:

The present invention relates to liposomes including an active substance, particularly a physiologically active substance.

Hitherto, in cases where a physiologically active substance such as a medicine is directly administered into a living body, there have been many occasions causing (1) immunological problems of proliferation of the antibody to the medicine within the body, (2) problems of augumented side effects of the medicine resulting from the uptake of the medicine by the tissues other than the target of the medicine, or inversely, (3) problems caused by the inability of the medicine of passing through the target tissue, and further (4) problems caused by the inability of the medicine of maintaining its activity owing to its degradation, inactivation, etc. by enzymes within the living body.

However, it is considered that the above-mentioned problems will be resolved by administering the physiologically active substance such as a medicine loaded on a carrier which is able to transfer the active substance directly into the target tissue within the living body while protecting the active substance.

From the above-mentioned viewpoint, Japanese Patent Application Laying Open No. 118826/74 (DE-OS-22 49 552) has recently proposed a liposome comprising a vesicle having a closed lamellar structure (micelle) composed of at least one

bimolecular layer formed by a compound, represented by the general formula, X-Y, wherein X represents a polar and hydrophilic group such as phosphate, carboxyl, amino, hydroxyl or choline and Y represents a non-polar and hydrophobic group such as alkyl, alkenyl or alkynyl, for example, a purified phospholipid such as lecithin, phosphatidylethanolamine, phosphatidylserine, etc. as a material for forming the membrane of the above-mentioned layer and containing a physiologically active substance dissolved in an aqueous solution within the vesicle of the liposome. Since the wall membrane which forms the liposome protects the active substance in the aqueous solution within the vesicle even under severe conditions, for instance, those in gastrointestinal tracts, the activity of the active substance is not spoiled even when the liposome is orally administered. In addition, since the permeability of the liposome to a tissue of living body changes depending on its particle size (diameter), it is possible to raise the permeability of the liposome to the tissue by adjusting the diameter of the liposome. Accordingly, the above-mentioned liposome has been given attention as a possible means of selectively supplying a physiologically active substance contained in the liposome into a specified tissue of a living body.

However, the above-mentioned wall membrane which forms the above-proposed liposome and is consisted of pure phospholipid has defects of lack of pliability and of insufficient mechanical strength. In addition, owing to the excessively large effluent

velocity of the physiologically active substance contained in the vesicle to the outside, the liposome is not necessarily satisfactory concerning its property of releasing the above-mentioned physiologically active substance slowly within the living body, that is, so-called the slow-releasing property. Particularly, the above-mentioned liposome has a defect that the effluent velocity of the above-mentioned active substance is highly raised at a temperature higher than the transition temperature of the wall membrane which forms the above-mentioned liposome.

In order to improve the strength of the wall membrane of the above-mentioned liposome, a method is well-known in which a steroid lipid such as cholesterol is admixed with the above-mentioned phospholipid as the material for forming the membraneous layer of the liposome. Although the strength of the wall membrane forming the liposome is somewhat improved by the proposed method, the so-called slow-releasing property is not so much improved.

In consideration of the above-mentioned situation, the inventor of the present invention, after studying the methods for supplying the liposome having a strong wall membrane and having a favorable slow releasing property of the physiologically active substance into the living body, has found that the wall membrane of the liposome which was formed by a phospholipid containing molecules of oily substance such as "crude lecithin" has a higher pliability and is more excellent in the slow-releasing property of the physiologically active substance into the living body as compared to the wall membrane of the

conventional and publicly known liposome formed by using purified phospholipid. Accordingly, it is surprising to see that the liposome formed by using a phospholipid containing molecules of oily substance has the above-mentioned specific properties.

Accordingly, the purpose of the present invention is to provide a liposome containing physiologically active substance, of which the wall membrane is strong enough and which has a favorable slow-releasing property of the above-mentioned active substance within the living body.

The other purposes of the present invention will be made clear from the following description:

BRIEF EXPLANATION OF THE DRAWINGS:

FIGURE 1 shows the schematic illustration of the liposome of the present invention and FIGURE 2 shows a relationship between the amount of molecules of oily substance contained in the membraneous material forming the liposome according to the present invention containing glucose in the aqueous solution within the vesicle of the liposome and the percentage of the amount of glucose entrapped within the vesicle to the total amount of glucose used for forming the above-mentioned liposome loading glucose (briefly expressed hereinafter as the rate of entrapment). FIGURE 3 shows a comparison of permeability of the above-mentioned glucose from the liposome of the present invention and from the liposome shown in Comparative Example. FIGURE 4 shows a comparison of the persistency of hypoglycemic

action of insulin in a living body, which is supplied by the liposome of the present invention containing insulin therewithin to that by the liposome of Comparative Example also containing insulin therewithin. FIGURES 5 to 8 show the slow-releasing property to an active substances included within the respective liposomes of the present invention.

Among them, FIGURE 5 shows the change of the amount of radio-isotope in blood with the passage of time after the subcutaneous injection of the liposome prepared by using the above-mentioned substance, however, radio-labelled, as the active substance, and FIGURE 6 shows the change of the amount of radio-isotope in urine with the passage of time after the same treatment as above. FIGURE 7 shows the change of the residual amount of radio-isotope, at the site where the free radio-labelled active substance was directly injected subcutaneously as it is, with the passage of time, and FIGURE 8 shows the change of the residual amount of radio-isotope, at the site where the liposome including the radio-labelled active substance within its vesicle was injected subcutaneously, with the passage of time.

DETAILED DESCRIPTION OF THE INVENTION:

The characteristic feature of the present invention is, on the formation of a liposome including an active substance within its vesicle surrounded by a micellar membraneous layer, the use a material comprising micellar membraneous layer of phospholipid, in which molecules of a fatty substance are present

as the wall membrane of the above-mentioned liposome.

Another characteristic feature of the liposome, including an active substance, formed by using the above-mentioned material according to the present invention is its W/O/W-type complex emulsified state as shown in FIGURE 1 and its excellent property of slowly releasing the above-mentioned active substance included within its vesicle to the outside when taken into a living body. In FIGURE 1, X represents a hydrophilic group, Y a hydrophobic group, P a vesicle containing an aqueous solution. A molecules of fatty substance and Q an aqueous solution outside of liposome, respectively.

The material for forming the liposome of the present invention, as has been described above, comprises phospholipid with which molecules of a fatty substance are mixed or to which they are bonded. The phospholipid used herein is not particularly limited provided it has been used as the material for a membraneous layer of the conventional liposome, for instance, a single compound of lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserin, phosphatidylinositol, sphingomyelin, cardiolipin, etc. or its mixture, and according to the necessity, a sterol compound such as cholesterol may be contained.

The fatty substance present in the above-mentioned phospholipid is triglyceride, wax or mineral oil or the mixture thereof, for instance, those selected from vegetable oil such as soy-bean oil, cotton seed oil and sesame oil, and also from

mineral oils of coal-origin or petroleum-origin.

Particularly preferable is the single use of crude lecithin or the use of a mixture of crude lecithin and other oily substance mentioned above.

The term "crude lecithin" herein used means a fraction which eluates with chloroform or a mixture of chloroform and methanol in a ratio of 100 : 1 to 3 : 2 when a component rich in phospholipid derived from substance such as yolk and soybean oil is fractioned by column chromatography using alumina as a column material, the fraction consisting of 97 to 80% by weight of pure lecithin and 3 to 20% by weight of oily substance, for instance, triglyceride and carotenoid.

In cases where the liposome of the present invention is prepared by using the above-mentioned crude lecithin as a material for membraneous layer, the content of the oily substance in crude lecithin is adjusted so that the content of the fatty substance in the thus formed wall membrane becomes to 3 to 20% by weight, preferably 5 to 15% by weight. In addition, care should be taken to keep the content of the fatty substance in the above-mentioned wall membrane not over 20% by weight or the formation of the wall membrane is impaired to have reduced yield of the liposome. On the other hand, in the case where the content of the fatty substance is less than 3% by weight, the above-mentioned object of the present invention cannot be attained.

By the way, on the formation of the liposome according to the present invention, sterols such as cholesterol and

ergosterol and a substance capable of changing the electrically loaded state of the surface of the liposome, for instance, phosphatidic acid, dicetyl phosphate or ganglioside of bovine brain for giving negative charge, or stearylamine for giving positive charge may be admixed with the material for membraneous layer as the third component. The amount of addition of the third component may be determined adequately according to the property of the phospholipid suitably, and usually is 0 to 10% by weight of the material for membraneous layer.

In the case of forming the liposome of the present invention while using a mixture of the above-mentioned phospholipid and the oily substance as a material for membraneous layer, the conventional method for preparing a liposome is possibly applied. For instance, a method in which a thin film is formed from the above-mentioned material and after bringing the thus formed membrane into contact with a continuous phase containing an active substance to form a dispersion by agitation, a supersonic vibration is applied on the dispersed system, a method in which after mixing a solution of the above-mentioned material for membraneous layer in a solvent insoluble in water with an aqueous solution containing the above-mentioned (water-soluble) active substance, the mixture is subjected to a supersonic treatment for forming a precursor of liposome and then the solution containing the precursor is subjected to ultracentrifugal treatment in the co-presence of an aqueous medium, or a method in which after coating the surface of glass-beads and the like

with the above-mentioned material for membraneous layer, the coated beads are mixed with an aqueous solution containing the above-mentioned active substance to disperse the coated beads into the solution is applicable.

The amount of the above-mentioned material for membraneous layer on forming the above-mentioned liposome is 1 to 500 mg/ml of the liquid in which the liposome is suspended.

The membraneous layer of the liposome obtained by the above-mentioned method is formed as a result of mutual action between the hydrophobic group possessed by the phospholipid in the material for membraneous layer which forms the liposome and the molecules of oily substance which are present in the material for membrane layer. Accordingly, the morphological state of the liposome of the present invention is to be said quite different substantially from the state of the conventional liposome comprising the micelle of purified phospholipid.

In addition, the oily substance as a constituent of the wall membrane of the liposome according to the present invention, on the formation of the liposome from the material for membraneous layer, brings about the improvement of the liposome-yield, and after forming the liposome, brings about the easiness in the process of separating the liposome, the uniformity of the particle size of the liposome and the improvement of pliability, the strength of the wall membrane of the liposome and the slow-releasing property of the active substance included in the vesicle of the liposome when administered within the living body.

The active substance, particularly the physiologically active substance used on the formation of the liposome of the invention is a medicine of which the side effect is to be reduced or a medicine of which the persistency within the living body is to be improved, and is exemplified by peptide hormons such as insuline, oxytocin, vasopression, adrenal-cortex stimulating hormon (ACTH), luteinizing hormon-releasing hormon (LH-RH), carcitonin, somatostatin, steroid hormons such as progesteron, follicular hormon, adrenal cortex hormon and other hormons such as prostaglandin, adenosine-3',5'-cyclicmonophosphate, anti-tumor agents such as chlorambutyl, streptozocin, methothorexate, 5-fluorouracil, sitosin arabinoside, mitomycin C, breomycin, polysaccharide derivative, antibiotics such as penicillin, cepharospolin, streptomycin, enzymatic preparations such as aminoglucosidase, invertase, etc.

The liposome according to the present invention comprises particles of 0.01 to 10 microns in average diameter with a narrow range of distribution of particle diameter, and those particles of 0.5 to 5 microns in average diameter are obtainable easily with a uniform diameter. The particles of liposome of the present invention are excellent in pliability, and they can be concentrated by a low speed-centrifugal treatment at 2,000 to 4,000 r.p.m. Moreover, the re-dispersion after concentration can be carried out only by a short time-shaking without necessitating an ultrasonic treatment to be recovered to the original state.

The liposome according to the present invention, as is

understood from its specific properties described above, is excellent in its capability of maintaining the active substance included within its vesicle as compared to the conventional liposome of the type of pure phospholipid micelle, and also its slow-releasing property to the active substance included therewithin is favorable. Accordingly, the liposome of the present invention is particularly suitable as a protecting agent for an unstable medicine within the living body, and also for a medicine which causes side effects by an inevitable excessive administration. By these reasons, the liposome according to the present invention is possibly utilizable as a medicine.

For instance, in the case where the liposome of the present invention is applied as an insulin injection, an intramuscular administration of the liposome of the present invention once two to seven days at a dose of 0.2 - 20 mg as insulin to an adult gives the same effect as that of the direct intramuscular administration of free insulin three times a day at a single dose of one third of 0.1 to 4.0 mg.

In cases where the liposome of the present invention is utilized as a medicine, its administration is possible via several routes such as percutaneous, subcutaneous, intramuscular, intraperitoneal, intravenal, intrarectal and topical, preferably by subcutaneous or topical administration. The amount of administration depends naturally to the method and the route of administration, the kind of active substance and the extent of treatment, however, usually it is 0.1 to 1 times as large as

the amount of the active substance directly administered per day, and further the interval of administrations can be extended.

In addition, the liposome of the present invention is possibly administered as an injection after dispersing the liposome into an aqueous physiological saline solution. Particularly, the liposome of the present invention containing a peptidic physiologically active substance including peptide hormons exhibits a remarkable effect when applied as a subcutaneous or intramuscular injection. Since peptidic physiologically active substances are generally degraded promptly in the living body, it is necessary to carry out frequent administration (injection) for the maintenance of the effect of the substance resulting not only in causing the heavier load on the patient but also causing the large fluctuation of the concentration of the active substance in blood. These situations are apt to reduce the effect of administration of the active substance and to cause side effects of the active substance.

Whereas, the liposome of the present invention, as are shown later in Examples, shows an excellent slow release of the included active substance on subcutaneous or intramuscular injection, and accordingly, the times of administration are possibly reduced materially with a maintenance of the uniform concentration of the peptidic physiologically active substance in blood, and accordingly, the effect of the active substance is fully exhibited with the suppression of its side effects.

According to the acute toxicity test of the above-

mentioned materials for forming the above-mentioned liposome of the present invention using rats via two routes of subcutaneous and intravenous injections, no toxic sign was observed on the treated rats until the dose rate reached 1,000 mg/kg concerning every material, the fact showing that the liposome of the present invention is safely applicable as a medicine from the viewpoint of the wall membrane of the liposome.

The followings are the concrete explanation of the present invention while referring to Examples.

Example 1:

A solution containing 100 mg of a commercial crude yolk lecithin (manufactured by Merck Company), 11.6 mg of cholesterol and 2.7 mg of stearylamine in 10 ml of chloroform was placed in a 25-ml round-bottomed flask set on a rotatory evaporator, and by distilling choloroform from the solution while rotating the evaporator at a temperature of 38°C under reduced pressure, a film was formed on the inner wall of the flask. Then, one milliliter of an aqueous 1% by weight solution of adenosine 3',5'-cyclic-monophosphate (hereinafter abbreviated as C-AMP) was added to the flask, and by shaking the flask for 30 minutes the film was exfoliated from the inner wall of the flask and the film was dispersed into the solution. By treating the dispersion with a supersonic treating machine (manufactured by Nippon Seiki Company, Model NS 200-2) for 20 min, a suspension-dispersion of particles of 1 to 2 microns in average particle

diameter was obtained. In the next step, an aqueous physiological saline solution of 6 times by volume of the above-mentioned suspension-dispersion was added to the suspension-dispersion, and the mixture was treated 3 times with centrifugal separation at 3,000 r.p.m. for each 10 min to separate completely the thus formed liposome, 1 - 1 and the residual solution of C-AMP which had not uptaken into the liposome. For comparison, four kinds of liposomes were prepared by using the materials for membraneous layer shown in Table 1, as Comparative Examples, 1 - 2, 1 - 3, 1 - 4 and 1 - 5 in the same procedures as in 1 - 1.

The rate of entrapment of C-AMP, that is, the percentage by weight of C-AMP collected within the vesicle of the liposome to the amount of C-AMP used, and the extent of release of C-AMP from the liposome after 24 hours of maintaining the liposome at a temperature of 37°C, that is, the residual percentage in the liposome, are shown in Table 1. As is seen in Table 1, by the use of the crude lecithin as the material for the membrane layer, the liposome of improved rate of entrapment and of the improved residual amount in the vesicle of the liposome as compared to those of the liposome prepared by using the conventional material for membraneous layer.

Table 1: Rate of Entrapment and Residual Percentage

Classification	Specimen	Composition of material for membrane (mg)	Rate of entrapment* (%)	Residual percentage of C-AMP within liposome vesicle**
Present invention	1 - 1	crude lecithin(+) 100 cholesterol 11.6 stearylamine 2.7	28	99.2
Comparative Examples	1 - 2	purified lecithin(++) 100 cholesterol 11.6 stearylamine 2.7	1.0	89.0
	1 - 3	purified lecithin 100 stearylamine 2.7	3.4	88.9
	1 - 4	purified lecithin 100 cholesterol 11.6	0.6	84.2
	1 - 5	purified lecithin 100	1.6	84.3

Note: (+) commercial material , (++) Commercial material

* Amount of C-AMP entrapped into the liposome expressed by percentage by weight of the amount of C-AMP used in preparation of the liposome.

** After keeping the liposome at 37°C for 24 hours.

In addition, commercial crude lecithin (manufactured by Merck Co.) and commercial purified lecithin (manufactured by Sigma Co.) had the following compositions, respectively:

unit: % by weight

Specimen	Phopholipid	Cholesterol	Oily substance
Crude lecithin(+)	93.8	1.1	5.1
Purified lecithin(++)	99.5	0.3	0.2

Example 2:

Liposomes were prepared by the same procedures as in Example 1 except for using one milliliter of an aqueous 20% by weight of glucose solution instead of an aqueous solution of C-AMP in Example 1 using each of the following materials for membraneous layer of the liposome shown in Table 2.

The amount of cotton seed oil as a component of the materials for the membraneous layer and the rate of collecting glucose in each liposome are correlated in Fig. 1. In addition, the residual amount of glucose in the liposome as compared to the initial amount of glucose in the liposome by percentage after maintaining for 24 hours at a temperature of 37°C was shown in Fig. 3, the determination of the above-mentioned glucose being carried out on Specimens of 2 - 7, 2 - 8 and 2 - 9.

From these figures, the superiority of the liposome of the present invention to the conventional liposomes is clearly recognized.

Table 2

Composition of Materials for Membraneous Layer

Classification	Specimen	Composition of Materials for Membrane	
		Base material (mg)	Cotton seed oil (mg)
Present invention	2 - 1	Crude lecithin(+) 100	0
	2 - 2	"	5
	2 - 3	"	10
	2 - 4	"	15
	2 - 5	"	20
Comparative example	2 - 6	"	40
	2 - 7	Purified lecithin(++) 100	0
Present invention	2 - 8	"	5
	2 - 9	"	10
	2 -10	"	15
	2 -11	"	20
Comparative example	2 -12	"	40

Note: (+) commercially available

(++) commercially available

Example 3:

By using the materials for membraneous layer having compositions shown in Table 3, while using the procedures described in Example 1, each film was formed on the inner wall of a round-bottom flask. After adding one milliliter of an aqueous citric acid-buffer solution containing 10 mg of insulin per 10 ml of the solution (pH of 2.3) to the flask, a suspension-dispersion of liposome particles of 1 to 2 microns in diameter was prepared by the same procedures as in Example 1. After leaving the suspension-dispersion at room temperature for 24 hours, it was treated one with 6 ml of an aqueous physiological solution and two times with a 6 : 1 mixture (by volume) of a physiological saline solution and an aqueous citric acid-buffer solution and subjected to centrifugal treatment to obtain a liposome. After adding an aqueous citric acid buffer solution to the thus prepared liposome to adjust the concentration of insulin in the mixture to 40 IU/ml (IU means an International standard unit), the mixture was subjected to the following experiment:

Table 3

Classification	Specimen	Composition of Material for Membraneous Layer			
Comparative Example	3 - 1	Commercial purified lecithin 100 mg	Cholesterol 11.6 mg	Stearylamine 2.7 mg	Cottonseed Oil 0 mg
Present Invention	3 - 2	as above	as above	as above	Cottonseed Oil 6 mg
do	3 - 3	as above	as above	as above	Cottonseed Oil 12 mg
do	3 - 4	Commercial crude lecithin 100 mg	as above	as above	Cottonseed Oil 0 mg

Experiment: Persistency test of insulin liposome within living bodies

Four groups of SD female rats artificially attacked by diabetes with the administration of streptozocine were respectively given subcutaneous injection of each liposome prepared as above, and their blood sugar was determined before and after the injection of the liposome. Fig. 4 shows the values of blood sugar with the lapse of time, the ordinate showing the percentage of the concentration of glucose in the blood after injection to the concentration of glucose in the blood before injection, and the abscissa showing the days after injection.

As is seen in Fig. 4, the persistency of insulin in the rat body (in other words, the fact that the reduced value of glucose was maintained for a long period of time) due to the application of the liposome of the present invention prepared by using crude lecithin inherently containing oily substance is far superior to that due to the application of the conventional liposome prepared by using purified lecithin not containing the oily substance.

Example 4:

The present example shows the transition of the active substance which has been included in the vesicle of the liposome after administration into the living body. For that purpose, the following experiments were carried out using a liposome including the tritium-labelled luteinizing hormon-releasing

hormon (LH-RH) as the active substance. The liposome was prepared by the following procedures:

Preparation of liposome:

The same film consisting of crude yolk-lecithin, chlosterol and stearylamine was prepared on the inner wall of a round-bottomed flask as in the first paragraph of Example 1. To the flask, 1 ml of an aqueous physiological saline solution containing tritium-labelled LH-RH (hereinafter referred to as ^{3}H-LH-RH) (250 μ Ci/7.3 μg, prepared by New England Nuclear Company) at a rate of 1 μg/ml was added, and after exfoliating and dispersing the film from the inner wall of the flask by shaking the flask for 30 min, the thus formed dispersion was treated by the supersonic treating machine (refer to Example 1) for 15 min to prepare a supension-dispersion of particles of 1 to 2 μ in average particle diameter. Six times by volume of an aqueous physiological saline solution as much as the suspension-dispersion was added to the suspension-dispersion and by subjecting the mixture to 2 times of centrifugal separation at 3,000 r.p.m. for each 10 min, the thus prepared liposome was completely separated from the solution of ^{3}H-LH-RH which has not been taken into the vesicle of the liposome.

The rate of collecting ^{3}H-LH-RH was 10% by weight. After adjusting the concentration of ^{3}H-LH-RH to 3.42 μCi/ml by the addition of an aqueous physiological saline solution, the solution was used for the following experiments.

Experiment 1:

The thus prepared liposome and the free ^{3}H-LH-RH not included in liposome were respectively injected subcutaneously into each ICR male mouse of body weight of 30 to 32 g of each group consisting of 3 animals at a dose rate of 0.34 μCi/animal, and the content of ^{3}H-LH-RH (expressed by the amount of the radio-isotope, RI) in the animal's blood was traced with the passage of time by collecting each 0.25 ml of blood specimen from each animal at a predetermined time interval after treating the specimen with a sample oxidizer (made by Packard Company) and determining the radioactivity by a liquid scintillation counter.

The results of determination were shown in Fig. 5, by taking the content of ^{3}H-LH-RH in the blood specimen after 15 min of the administration as 100.

As is seen in Fig. 5, the hormon included in the vesicle of the liposome prepared according to the present invention is slowly released within the living body.

Experiment 2:

The above-mentioned liposome and the free ^{3}H-LH-RH were respectively injected subcutaneously to each SD male rat of each group consisting three animals, and the excreted amount of the radio-isotope in urine and feces was traced with the passage of time. The rate of administration was 0.68 μCi/animal, and the excreted amount of the radio-isotope was determined by scintillation counting the specimen after diluting the urine

specimen with distilled water to 100 ml, or drying the feces and oxidizing it.

The results are shown in Fig. 6 by taking the administered amount as 100, and integrating the excreted amount to the time of determination. No radio-isotope was detected in the faces.

From Fig. 6, it will be recognized that the hormon included in the liposome prepared according to the present invention is slowly released from the liposome within the living body.

Experiment 3:

The same liposome as used in Experiments 1 and 2, and the free ^{3}H-LH-RH were respectively injected subcutaneously into each ICR male mouse weighing 30 to 32 g of each group consisting 2 mice, and the residual amount of the radio-isotope at the site of injection was traced with the passage of time after the administration of 0.165 μCi. The determination of the residual amount of the radio-isotope was carried out by taking out the region of injection and after dissolving the region into SOLUENE* (supplied by Packard Company) using scintillation counting. The results are: (1) as is shown in Fig. 7, after injecting the free ^{3}H-LH-RH, the residual amount of RI decreased remarkably in a short time after injection, whereas (2) as is shown in Fig. 8, it showed an extremely slow reduction when administered in the state of inclusion in the vesicle of the liposome.

According to the results of the above-mentioned

*Trade Mark

experiments 1 to 3, the slow releasing property of the active substance included in the vesicle of the liposome according to the present invention has been verified.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A liposome comprising a wall membrane, having a structure in which molecules of at least one oily substance are present within a bimolecular layer of phospholipid in an amount of 3 to 20% by weight of said phospholipid, and at least one physiologically active substance included in the vesicle of said liposome.

2. The liposome according to claim 1, wherein said phospholipid is lecithin.

3. The liposome according to claim 1, wherein said oily substance is one or a mixture of at least two oily substances selected from the group consisting of mineral oils, waxes and triglycerides.

4. The liposome according to claim 1, wherein said wall membrane comprises crude lecithin containing at least 3% by weight of at least one oily substance.

5. A composition for forming liposome, comprising a phospholipid, at least one oily substance in an amount of 3 to 20% by weight of said composition and at least one physiologically active substance.

6. The composition according to claim 5, wherein said oily substance is one or a mixture of at least two oily substances selected from the group consisting of mineral oils, waxes and triglycerides.

FIG. 1

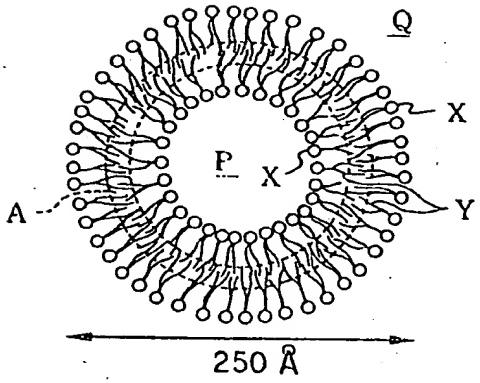
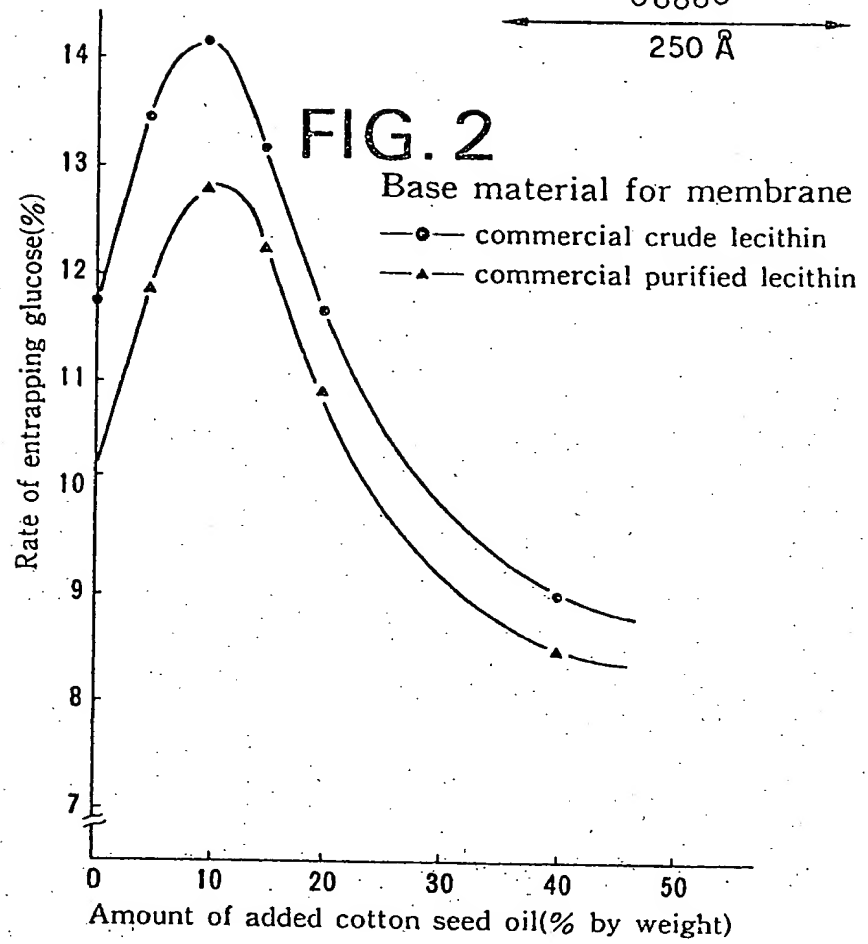


FIG. 2

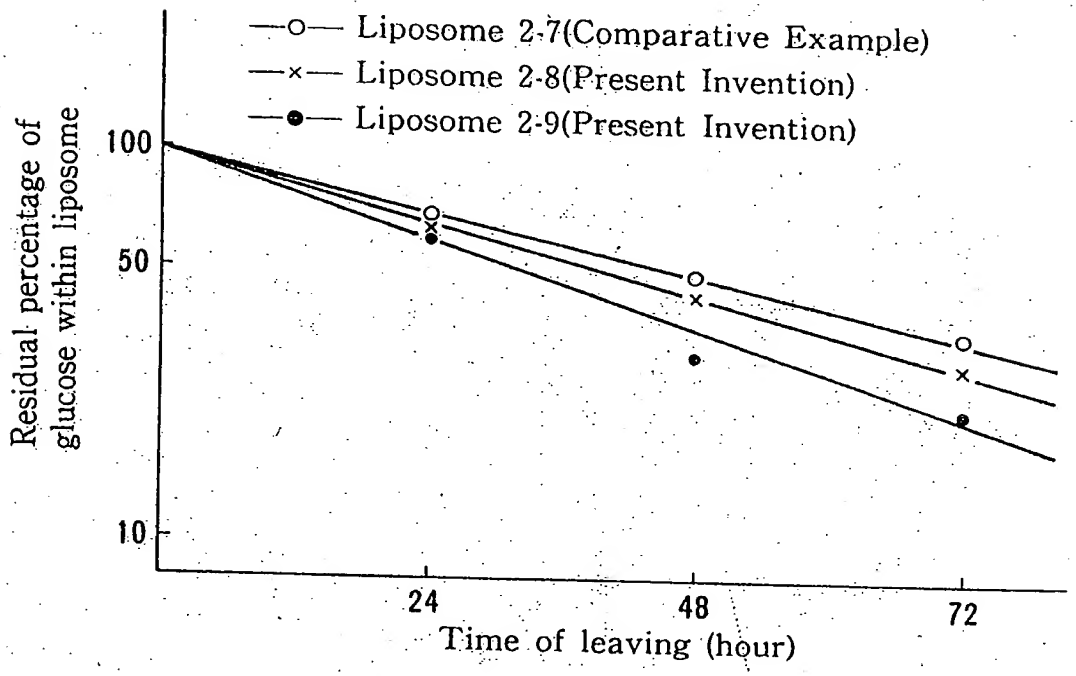


A

Scott & Aylen

1-2

FIG. 3 Permeability of Glucose



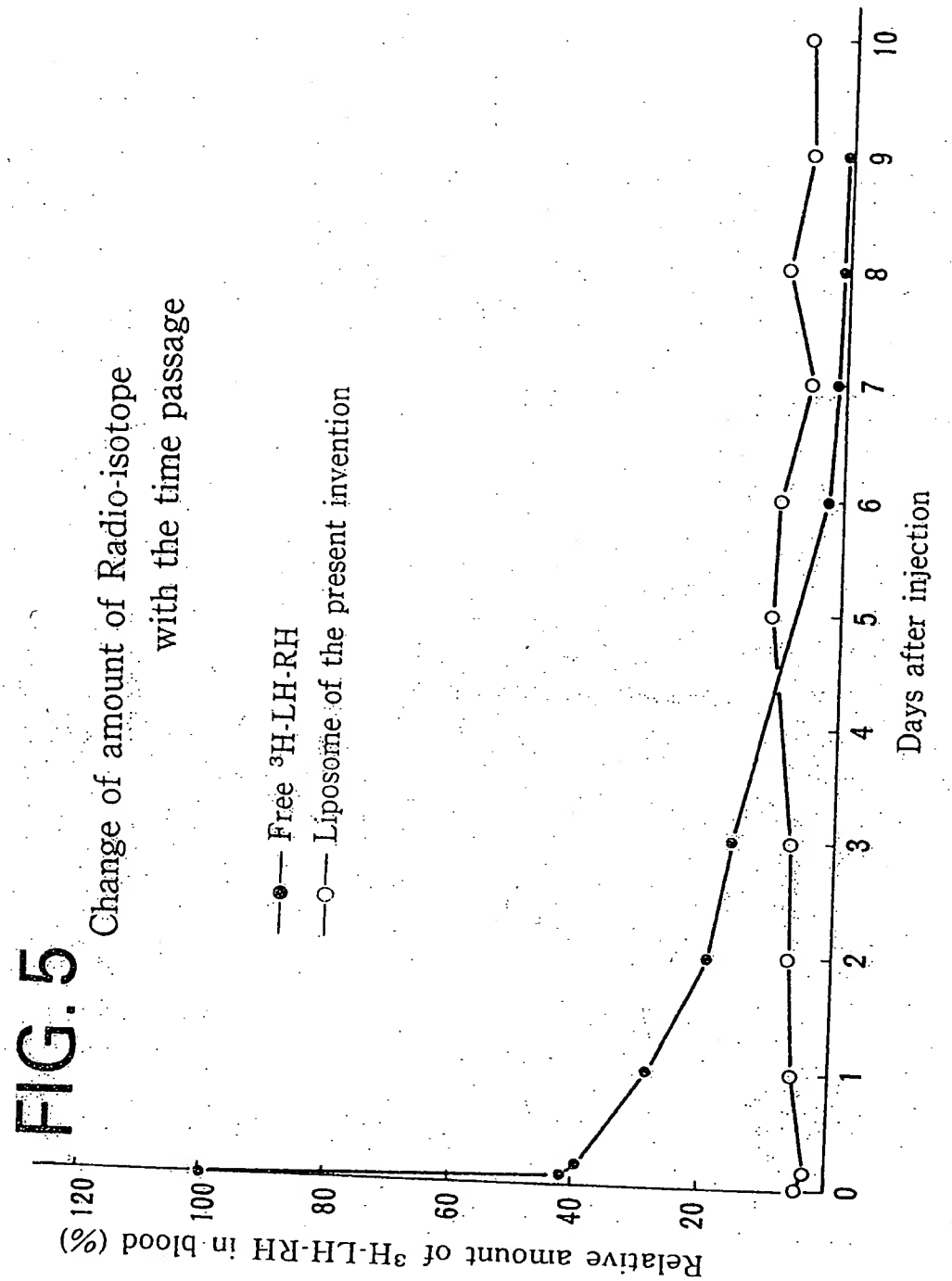
Scott & Aylen

FIG. 4 Persistency of Active Substance (Insulin) within the living body

Scott & Aylen

FIG. 5

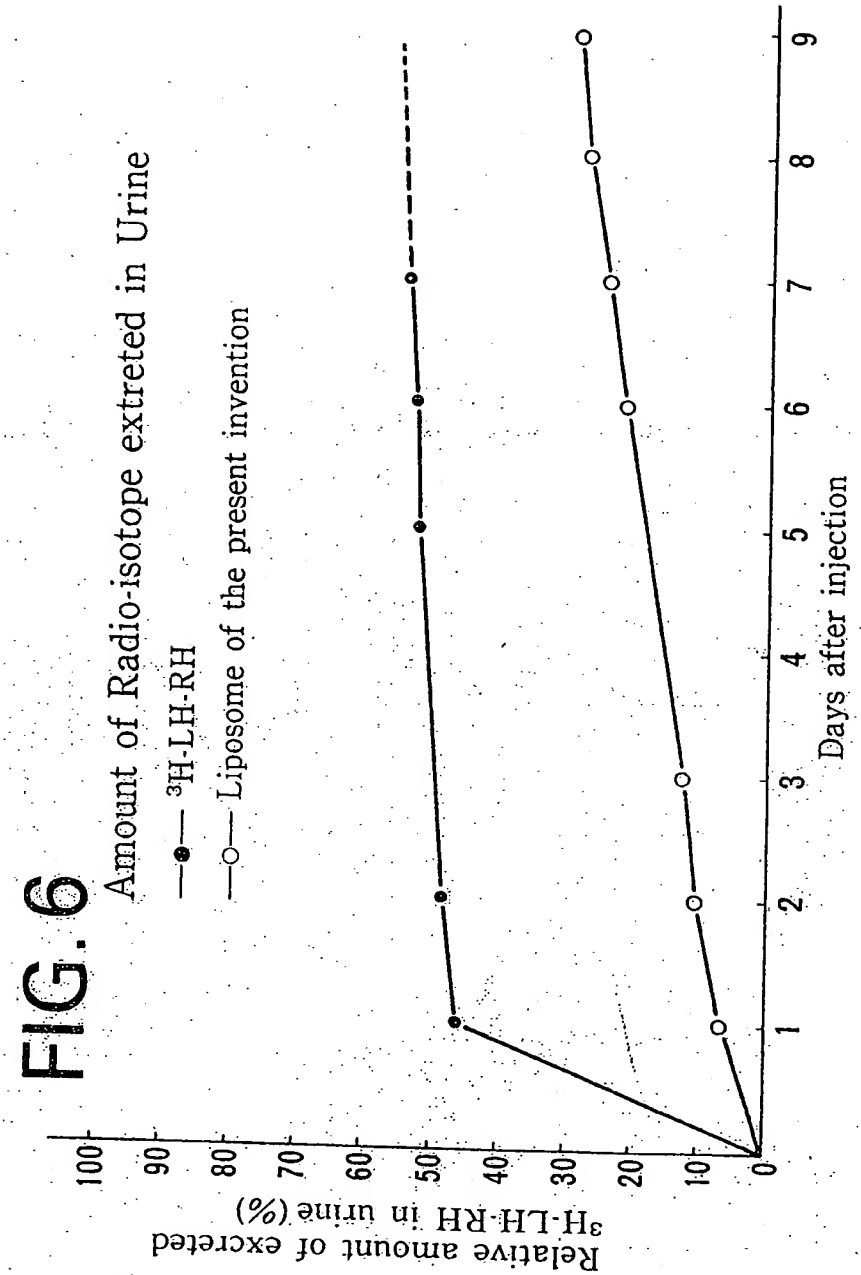
Change of amount of Radio-isotope with the time passage



Scott & Aylen

7-5

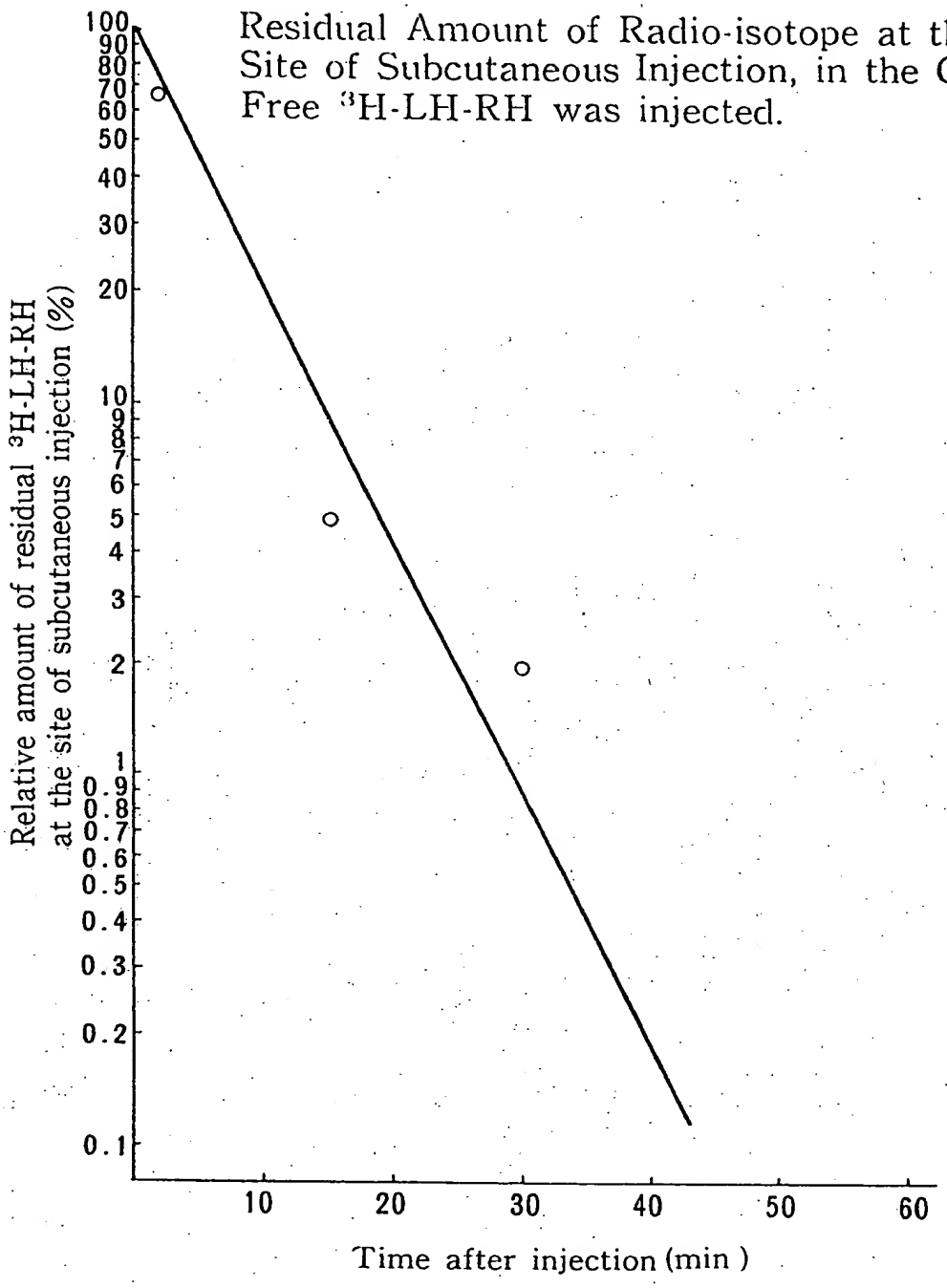
FIG. 6



Scott & Aylen

FIG. 7

Residual Amount of Radio-isotope at the Site of Subcutaneous Injection, in the Case Free ^{3}H-LH-RH was injected.



Scott & Aylen

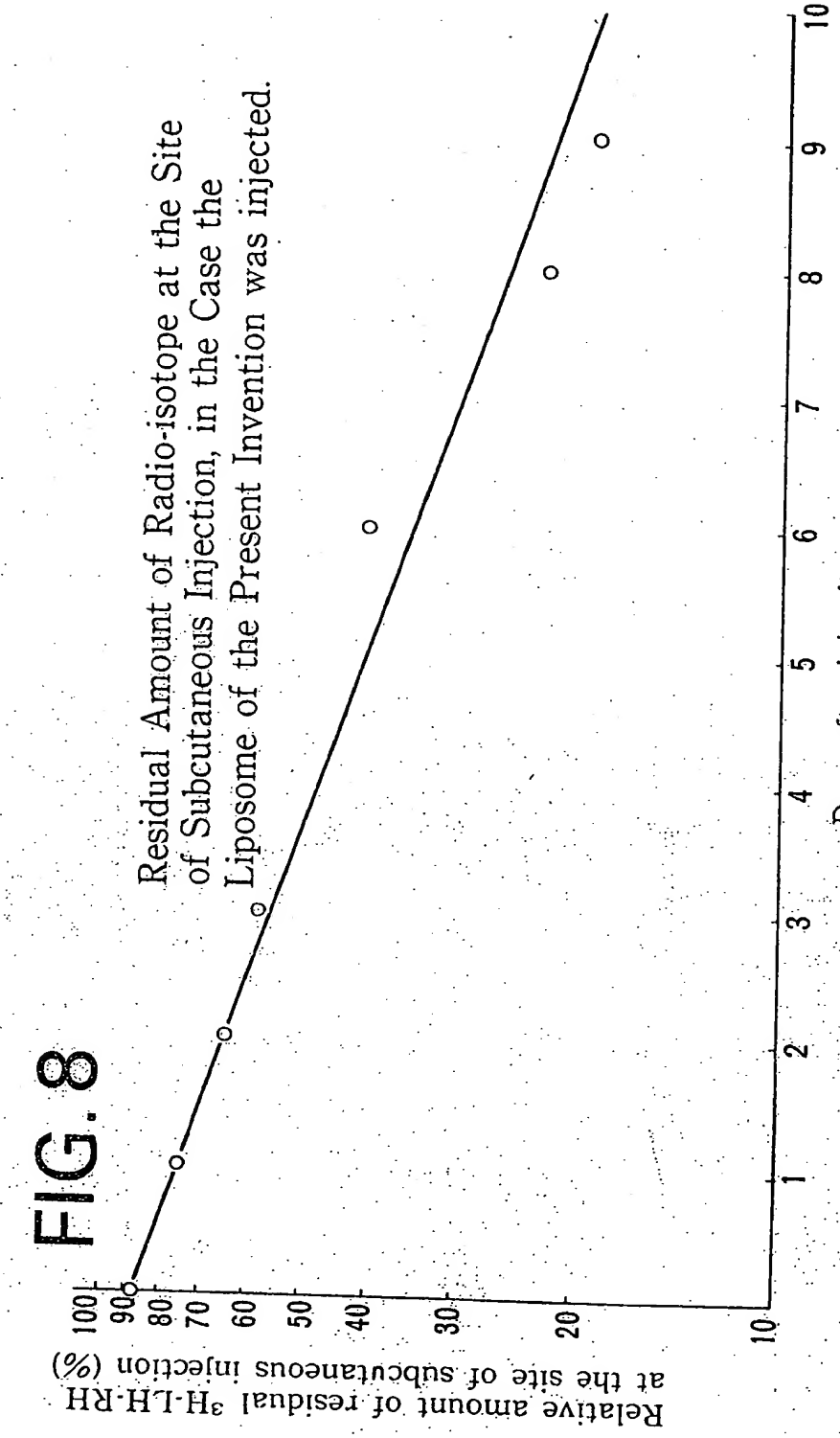


FIG. 8

Scott & Aylen